Studies on Pathogenesis of Cholesterol Gallstones, Especially with Respect to Behaviour of Cholesterol Metabolism

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Studies on Pathogenesis of Cholesterol Gallstones, Especially with Respect to Behaviour of Cholesterol Metabolism

by

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I. INTRODUCTION

Despite studies which have been undertaken by many investigators, the etiology of cholelithiasis still remains obscure.

In our laboratory, HIKASA et al. have investigated many aspects of essential fatty acid (EFA) metabolism from the stand point of surgical physiology. They demonstrated that EFA esterified with cholesterol, especially tetraenoic acid in the adrenals of rats, play an importent role in the catabolic metabolism of cholesterol to corticosteroid hormones. MARUYAMA and YOSHINAGA demonstrated that the degradation of cholesterol to bile acids might also be influenced by EFA and vitamin B_6 in the liver in the same way as in the adrenals, because rats deficient in EFA and/or vitamin B_6 had lower levels of bile acids and higher ratios of cholesterol to bile acids and of dihydroxycholanic acid to trihydroxycholanic acid in the bile. HIRANO found that the amount of EFA, especially of tetraenoic acid, was decreased in the liver of patients with gallstones in comparison with those of other diseases.

This paper describes investigations of cholesterol metabolism in relation to gallstone formation.

II. EXPERIMENTAL MATERIALS AND ANIMALS

A) Gallstones obtained from patients admitted to Kyoto University Hospital and Toyosato Hospital were analysed. The 33 stones, which were composed mainly of cholesterol or calcium bilirubinate, were ground into powder and desiccated uatill they reached a constant weight.

B) The animals used in this experiment were male albino rats of the Wister strain supplied by the Animal Center of Kyoto University, and golden hamsters (Mesocricetus auratus) obtained from the Central Laboratory of Experimental Animals. These animals had received a rat chow (Niippon Haigo Shiryo K.K.) and water ad libitum. The rats were divided into eight groups and housed individually in wire-screen floored cages at a constant temperature of 20°C. Hamsters were divided into five groups similarly. Thereafter, they were fed several synthetic diets for 5-6 weeks, as shown in table 1. As the source of EFA, purified sesame oil containing 48.9% linoleic acid was used. Inositol and biotin were abcent from the vitamin mixture. Silica gel was produced from the reaction, $Na_4SiO_3 + 4HCl \rightarrow H_4SiO_3 + 4NaCl$. Excess NaCl was removed by dialysis with cellophane,

Table 1 Composition of Various Synthetic Diets

Diet / % w/w	Starch fat	Starch fat-free	Starch fat Mg free	Starch fat Mg free V.B_6 free	Starch fat Excess Silica	Sucrose fat	Sucrose fat free	Sucrose fat V.B_6 free
Potato-Starch	63.5	73.5	63.5	63.5	63.5	—	—	—
Sucrose	—	—	—	—	—	63.5	73.5	63.5
Vitamin-free Casein	20	20	20	20	20	20	20	20
Salt Mixture	5	5	5*	5*	5	5	5	5
Vitamine Mixture	1	1	1	1**	1	1	1	1**
Sesame Oil	10	—	10	10	10	10	—	10
Choline Chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Silica	—	—	—	—	+	—	—	—

* exclude $MgSO_4$ ** exclude pyridoxine HCl

Table 2

Vitamin Mixture per 1g	
Vit. A	2500 I.U.
Thiamin Nitrate	1.0mg
Riboflavin	1.5mg
〔Pyridoxine HCl〕	〔1.0mg〕
Cyano-cobalamin	1.0γ
Ascorbic Acid	37.5mg
Calciferol	200 I.U.
dl-α-Tocopherol	1.0mg
Vit. K	0.2mg
Nicotinic Acid Amide	10.0mg
Cal. Pantothenate	2.5mg
Folic Acid	0.5mg

Table 3

Salt Mixture per 1kg	
NaCl	46.3g
NaH_2PO_4	92.0g
K_2HPO_4	253.0g
$CaH_4(PO_4)_2 \cdot H_2O$	143.0g
Cal. Lact.	369.0g
〔$MgSO_4$〕	〔70.4g〕
KI	26.3g

the silica gel was added to the basal diet. Except for the ion free water given to the group fed magnesium-deficient diets, food and water were provided ad libitum. The magnesium-deficient diet contained only 20.5 p. p. m. of magnesium, whereas the other diets contained 380-400 p. p. m.

During the course of the study, these animals exhibited the characteristic signs induced by each diet.

III. EXPERIMENTAL METHODS

All animals were sacrificed by bleeding from the aorta under intraperitoneal anesthesia at the end of their feeding period. Liver slices were immediately excised and weighed and analysed biochemically.

1) Extraction of lipids

To powdered human gallstones and homogenized liver were added ca. 20 volumes of ethanol ether solution (BLOOR's solution, ethanol : ethyl ether 3 : 1 V/V) or acidic BLOOR's solution (ethanol : ethyl ether : HCl 300 : 100 : 21 V/V) and 0.1 cc of 0.1% hydroquinone in ethanol to prevent oxidation. After standing overnight at room tempera-

ture, the residue was removed by glass filter No. 3G4, and the lipids were extracted by shaking 3 times with petroleum ether.

2) Fractionation of the lipids

Cholesterol esters were separated from the other lipids by column chromatography, as in the method of HIRSH and AHRENS. Though the lipids were extracted far more effectively with acidic than with non-acidic BLOOR's solution, tbe latter was used in this fractionation, because lipid fractionation by column chromatography on silicic acid is often disturbed by HCl.

3) Saponification

Total cholesterol and fatty acids, which were extracted with acidic BLOOR's solution, and esterified cholesterol and fatty acids extracted with non-acidic solution were each saponified with 2 % ethanolic KOH at 40°C for 90 minutes.

4) Determination of cholesterol

Cholesterol was measured at 620 mμ in a photoelectric spectrophotometer, Shimadzu Type QB-50, by the method of ABELL et al.

5) Separation of fatty acids

After saponification, fatty acids were methylated by means of diazomethane. The methylesters of fatty acids were analysed in a Shimadzu Gaschromatograph, Model GC 1-B equipped with a hydrogen flame ionization detector. A 150cm U shaped stainless steel column of 6 mm i.d. was used in which was packed 25% diethylene glycol succinate coated on Shimalite-Q, 60-80 mesh. The flow rate of nitrgen as the carrier gas was 36 ml/min. at an inlet pressure of 3 kg/cm. The column bath was kept at 210°C, the detector bath at 230°C and the inlet at 300°C. The main fatty acids were identified by comparison with authentic samples supplied by the Nutritional Biochemical Corp. of Cleveland, Ohio, U. S. A.. The other fatty acids were identified by their carbon numbers and retention times.

Quantification of chromatographed fatty acids was carried out by the product of the relative retention time and the height of each peak. The values obtained differed little from those obtained by triangulation. The fatty acid composition was represented as area per cent which agreed well with their weight per cent.

6) Determination of absolute quantities of fatty acids

Two different procedures were used for quantification of fatty acids.

a) Petroleum ether extracts of free fatty acids were evaporated in a water bath at 40°C under a stream of nitrogen gas and weighed in the microbalance after desiccation for 24 hours. The absolute amount of fatty acids contained in human gallstones were weighed by this method.

b) The liver content of fatty acids was too little to weigh, so the alkaline isomerization method was used for the determination of absolute amounts of hepatic fatty acids. Tetraenoic acid, a polyunsaturated fatty acid, was measured by ultra-violet spectrophotometry after alkaline isomerization for 20 minutes at 180 ± 1 C using 21 ± 0.5 % KOH in ethylene glycol acocrding to the method of JINDO (a modification of that of HOLMAN and HAYES). The values of tetraenoic acid obtained by this method were applied to those of arachidonic acid and docosatetraenoic acid, represented as percentages by gas

liquid chromatographic separation. The amounts of fatty acids were calculated from these two values.

The amounts of cholesterol and fatty acids were represented as mg/100g of liver wet weight.

IV. RESULTS

1) Growth rate, skin lesions and other manifestations in animals on various diets.

In young animals, growth was far more rapid when starch was the source of carbohydrate than when sucrose was. Hamsters on a sucrose fat-free diet developed coarse fur, occasional diarrhea and anal prolapse. Animals fed starch fat-free diets grew less than the controls and also had coarse fur and skin lesions to some degree. Rats fed a diet deficient in magnesium began to have hyperemia of the ears and developed diarrhea and excitability 2-3 weeks after the start of the diets. Skin lesions then appeared as tiny scabs on the face which occasionally enlarged or developed into open bleeding sores. Animasl fed sucrose diets deficient in vitamin B_6 grew more slowly than the controls but developed no significant skin lesions during the experimental feeding period. Rats fed starch diets with silica grew normally and seemed to deposit moderate amounts of body lipids ; no skin lesions or other abnormalities were observed.

2) Cholesterol level in livers of experimental animals.

The levels of total and esterified cholesterol and the ratio of esterified cholesterol to tatal cholesterol in the liver were generally higher in hamsters than in rats. Total cholesterol levels were highest both rats and hamsters on fat-free sucrose diets. The groups on fat-rich starch diets showed the lowest levels of total cholesterol. The levels were, in general, higher in all animals on fat-free diets than in those on fat-rich diets. In hamsters the difference in total cholesterol between those on fat-rich and those on fat-rich diets was far greater than the differnce between those on starch and those on sucrose diets, but in rats it was vice versa. Rats fed diets deficicient in magnesium or deficient in pyridoxine showed insignificant changes in their total cholesterol levels.

The esterified cholesterol levels in the liver of these animals showed the same tendency as the total cholesterol lavels. except that in pyridoxine-deficient animals the levels were lower than in the controls and the ratio of esterified cholesterol to total cholesterol was

Table 4 Cholesterol Concentration in the Liver of Rats (mg/100g wet weight of liver)

Rats fed various diets	Total cholesterol	Esterified cholesterol	Est. chol. / Tot. chol.
Starch fat	244.5±57*	34.2±5.0	0.14
Starch fat free	275 ±14	34.5±1.8	0.13
Sucrose fat	473 ±45	36.9±4.6	0.078
Sucrose fat free	494 ±70	54.3±4.1	0.11
Sucrose fat Vit. B_6 free	290.5±56	19.3±4.3	0.066
Starch fat Mg free	309 ±29	33 ±2.7	0.107
Starch fat Mg free Vit. B_6 free	242 ±30	39 ±1.2	0.163
Starch fat excess Si	255 ±26	41.4±1.7	0.179

* : Standard deviation

Table 5 Cholrsterol Concentration in the Liver of Hamsters (mg/100g wet weight of liver)

Hamsters fed various diets	Total cholesterol	Esterified cholesterol	Est. chol. / Tot. chol.
Starch fat	334 ±60*	101 ±20	0.301
Starch fat free	528.8±42	156 ±76	0.295
Sucrose fat	456.6±61	103 ±31	0.226
Sucrose fat free	629.0±78	182 ±32	0.336
Sucrose Vit. B_6 free	342.2±53	32.2±15	0.088

* : Standard deviation

also low. Esterified cholesterol levels were much higher in hamsters fed fat-free diets than in those fed fat-rich diets. Esterified cholesterol levels were lower in rats than in hamsters. (Table 4, 5)

3) Fattty acid level in the livers of experimental animals.

Total fatty acid levels in the liver were almost the same in rats as in hamsters and were highest in the fat-free sucrose diet groups. They varied less among the various diet groups than did the total cholesterol levels. However, they were very low in animals on fat-rich starch diets and moderately low in magnesium-deficient animals. The changes in esterified fatty acid levels were similar to those of esterified cholesterol, being highest in the fat-free diet groups and lowest in the pyridoxine-deficient groups.

The per cent of liver arachidonic acid, especially esterified arachidonic acid, was much lower in hamster than in rats, even in those on fat-rich diets. The levels of linoleic acid and arachidonic acid were much lower in the fat-free diet group than in the fat-rich diet group. On the contrary, saturated fatty acids, palmitoleic, oleic and eicosatrienoic acids increased as expected. In hamsters the arachidonic acid level was lowest 2 weeks after the start of the fat-free sucrose diet, and trienoic acid appeared at 4-5 weeks. The

Table 6 (a) Fatty Acid Composition of Total Lipid and of Cholesterol Ester in the Liver of Rats

Diets / Types / Fatty acids	Starch fat		Starch fat free		Sucrose fat		Sucrose fat free	
	Total	Ester	Total	Ester	Total	Ester	Total	Ester
14 : 0	0.2±0.0*	0.6±0.1	0.3±0.1	1.5±0.4	0.6±0.3	1.2±0.3	0.9±0.1	1.9±0.1
16 : 0	14.9±1.4	25.5±2.5	18.4±0.3	27.6±1.2	18.2±1.3	23.4±2.5	25.3±1.9	28.4±3.2
16 : 1	1.17±0.3	2.2±0.6	6.1±0.5	6.7±0.5	1.5±0.3	6.16±2.3	11.7±1.8	7.2±1.0
18 : 0	17.6±2.1	23.8±4.8	14.3±3.1	28.1±6.5	18.6±0.7	11.0±3.9	3.2±0.5	29.3±1.2
18 : 1	15.2±0.7	20.3±4.0	24.8±4.0	16.3±0.5	17.0±0.9	31.0±3.6	52.6±3.2	17.6±1.0
18 : 2	14.9±2.9	13.6±3.5	5.2±1.3	1.8±0.4	12.9±0.7	16.4±4.5	2.0±0.0	1.0±0.0
18 : 3	0.5±0.3	0.2±0.0	0.4±0.1	0.7±0.0	0.2±0.2	0.5±0.1	0.2±0.0	0.7±0.0
20 : 0	0.1±0.0	—	0.4±0.1	—	0.1±0.0	0.1±0.0	0.1±0.0	—
20 : 3	—	—	6.3±2.3	1.7±0.7	—	—	2.3±0.1	2.5±0.1
20 : 4	27.3±1.1	12.3±1.7	11.4±1.1	3.7±0.2	25.5±1.3	10.3±1.7	1.8±0.5	2.2±0.1
22 : 0	—	—	0.3±0.2	—	—	—	—	0.2±0.0
22 : 4	0.5±0.2	—	—	—	1.4±0.1	—	—	—
22 : 5	1.6±0.8	—	0.7±0.0	—	0.3±0.0	—	—	—
22 : 6	2.2±0.9	—	3.2±0.2	—	2.3±1.0	—	2.3±0.1	1.9±0.0

* : Standard deviation

Table 7 (b) Fatty Acid Composition of Total Lipid and of Cholesterol Ester in the Liver of Rats

Diets / Types / Fatty acids	Starch fat Mg free		Starch fat Excess Si		Starch Mg free V. B_6 free		Sucrose fat V. B_6 free	
	Total	Ester	Total	Ester	Total	Ester	Total	Ester
14 : 0	0.3±0.1*	0.5±0.1	0.3±0.1	0.5±0.1	0.2±0.1	0.6±0.1	0.2±0.0	0.9±0.5
16 : 0	15.9±1.7	20.9±3.3	15.9±1.7	20.9±3.3	16.5±1.5	28.1±4.	19.1±0.9	30.5±4.0
16 : 1	1.3±0.8	1.8±0.8	1.2±0.8	1.7±0.8	1.1±0.4	2.7±1.1	1.5±0.3	4.1±0.7
18 : 0	21.9±2.6	21.3±7.7	21.9±2.6	21.2±7.7	21.5±2.8	22.5±7.3	21.5±1.2	11.4±0.7
18 : 1	13.7±3.2	22.5±5.2	13.7±3.2	22.5±5.2	14.3±2.5	16.1±1.7	15.4±1.1	26.9±2.3
18 : 2	11.7±1.9	19.4±5.5	11.7±1.9	19.3±5.5	11.8±1.2	12.3±2.0	16.9±1.0	13.9±2.0
18 : 3	0.5±0.0	0.3±0.1	0.5±0.3	0.3±0.1	0.7±0.1	0.3±0.1	0.3±0.1	—
20 : 0	0.1±0.0	—	0.1±0.0	—	—	—	0.1±0.0	—
20 : 3	—	—	—	—	0.3±0.0	—	—	—
20 : 4	27.4±4.4	12.2±4.2	27.3±4.4	12.2±4.2	24.7±4.1	11.7±4.6	19.9±2.5	10.1±1.2
22 : 0	—	—	—	—	—	—	—	—
22 : 4	0.6±0.0	—	0.6±0.0	—	0.7±0.1	—	0.2±0.0	—
22 : 5	0.4±0.0	—	—	—	1.2±0.1	—	0.12±0.0	—
22 : 6	2.2±1.4	—	2.2±1.4	—	2.0±0.2	—	4.7±0.5	—

* : Standard deviation

Table 8 Fatty Acid Composition of Total Lipid and of Cholesterol Ester in the Liver of Hamsters

Diets / Types / Fatty acids	Starch fat		Starch fat free		Sucrose fat		Sucrose fat free		Sucrose fat V. B_6 free	
	Total	Ester	Total	Ester	Total	Ester	Total	Ester	Total	Ester
14 : 0	0.45±0.3*	0.7±0.1	0.8±0.1	0.7±0.3	0.8±0.1	0.1±0.0	0.6±0.1	1.1±0.6	0.2±0.0	0.1±0.0
16 : 0	20.9±1.6	23.9±3.2	26.1±3.4	18.5±2.0	22.9±3.2	19.0±2.0	18.1±1.1	19.1±2.3	20.8±0.7	24.6±5.8
16 : 1	1.1±0.3	1.8±0.2	8.3±1.0	10.1±1.9	4.14±0.8	5.2±0.8	6.5±1.8	7.8±1.6	1.1±0.0	3.5±1.0
18 : 0	19.4±1.6	20.7±6.1	5.1±0.3	7.5±1.0	6.35±0.5	21.0±3.1	7.6±1.7	6.4±2.6	15.6±3.1	14.3±2.6
18 : 1	17.4±2.1	27.7±1.0	47.5±5.9	50.6±1.3	39.9±4.1	30.1±2.3	41.9±2.4	51.7±4.8	20.2±1.6	35.2±3.6
18 : 2	22.6±1.0	20.9±3.3	3.3±0.8	3.9±0.9	19.7±2.1	21.2±3.0	5.1±2.4	1.18±1.9	21.1±3.1	18.0±3.8
18 : 3	—	—	—	0.1±0.0	—	—	—	0.1±0.0	0.2±0.0	0.2±0.0
20 : 0	0.13±0.0	—	—	1.2±0.0	—	—	0.4±0.0	1.0±0.0	0.6±0.4	0.2±0.0
20 : 3	0.16±0.0	—	2.05±0.1	2.2±0.1	—	—	8.3±3.9	1.7±0.6	0.4±0.1	—
20 : 4	13.4±1.6	4.2±1.0	5.8±0.9	1.0±0.0	5.72±0.7	3.0±0.5	4.4±0.9	0.6±0.0	11.4±0.6	2.0±1.2
22 : 0	0.4±0.0	—	0.2±0.0	—	0.1±0.0	—	0.9±0.0	0.1±0.0	—	—
20 : 4	—	—	—	—	—	—	1.5±0.1	—	0.2±0.0	—
20 : 5	—	—	—	—	—	—	—	—	2.0±0.5	—
20 : 6	2.3±0.0	—	1.1±0.0	0.1±0.0	0.5±0.0	—	3.3±0.7	—	5.2±0.1	—

* . Standard deviation

linoleic acid level changed more extensively and far more quickly than the arachidonic acid level in animals on both fat-free and fat-rich diets.

On the other hand, in those on the sucsose diet the ratio of linoleic and arachidonic acid to saturated fatty acids and oleic acid was lower than in those on the starch diet. Thus, the net increase of total fatty acids in the animals on sucrose diets appeared to depend on these saturated fatty acids and oleic acid, each of which could be synthesized from other food stuffs in vivo. These findings suggest that these animals were relatively

Table 9 Fatty Acid Concentration in the Liver of Rats (mg/100g wet weight of liver)

Rats	Total fatty acid	Esterified fatty acid with cholesterol	Total fatty acid 20 : 4/18 : 2	Est. fatty acid 20 : 4/18 : 2	Total fatty acid $\frac{20:4+18:2}{Sat.+18:1}$	Est. fatty acid $\frac{20:4+18:2}{Sat.+18:1}$
Starch fat	1513±301*	32.3±4	1.82	0.90	0.88	0.37
Starch fat free	2200±270	39.2±2	2.18	2.06	0.26	0.08
Sucrose fat	2065±700	30.9±12	1.96	0.63	0.68	0.40
Sucrese fat free	2370±195	49.1±25	0.88	2.20	0.05	0.02
Sucrose fat V.B_6 free	1600±520	18.7±5	1.18	0.72	0.66	0.35
Starch fat Mg free	1250±270	28 ±4	1.84	1.29	0.70	0.29
Starch fat Mg freeV.B_6 free	2190±340	11.0±3	1.66	0.95	0.75	0.36
Starch fat excess Si	2200±103	42.4±5	1.85	0.63	0.81	0.48

* : Standard deviation

Table 10 Fatty Acid Concentration in the Liver of Hamsters (mg/100g wet weight of liver)

Hamsters	Total fatty acid	Estrified fatty acid with cholesteral	Total fatty acid 20 : 4/18 : 2	Est. fatty acid 20 : 4/18 : 2	Total fatty acid $\frac{20:4+18:2}{Sat.F.A.+18:1}$	Est. fatty acid $\frac{20:4+18:2}{Sat.F.A.+18:1}$
Starch fat	1239±610*	52±22	0.59	0.21	0.62	0.34
Starch fat free	1920±320	88±46	1.75	0.25	0.11	0.06
Sucrose fat	2200±200	63±31	0.29	0.14	0.33	0.33
Sucrose fat free	2300±230	87±29	0.88	0.16	0.12	0.06
Sucrose fat V.B_6 free	1705±180	31±18	0.55	0.11	0.57	0.26

* : Standard deviation

deficient in essential fatty acids. In the magnesium-deficient animals there was a slight decrease in linoleic acid but not in arachidonic acid levels; however, the influence of diarrhea on fat absorption could not be disregarded. In rats fed a diet containing silica, the level of arachidonic acid was elevated. (Table 6, 7, 8, 9, 10)

4) Analysis of human gallstones.

Calcium bilirubinate gallstones contained approximately five times the amount of total fatty acids as cholesterol stones. Both cholesterol and calcium bilirubinate stones contained very small amounts of essential fatty acids and relatively large amounts of other fatty acids. The fatty acid composition of these two types of gallstones was similar. (Table 11)

In summary, the levels of total cholesterol, total fatty acids, esterified cholesterol and

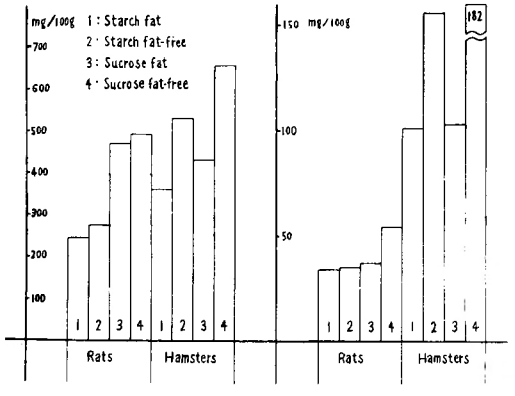
Table 11 Fatty Acids and Cholesterol in Human Gallstones

	Bilirubin 13 Stones	Cholesterol 21 Stones
Total fatty acid (mg/100g of stone)	4600	980
14 : 0 (%)	1.2	1.5
16 : 0	56.4	50.3
16 : 1	0.9	0.8
18 : 0	10.5	9.6
18 : 1	13.2	11.7
18 : 2	9.9	12.8
18 : 3	—	—
20 : 0	0.9	3.5
20 : 3	1.1	0.9
20 : 4	1.7	2.2
22 : 0	0.7	0.7
22 : 4	—	—
22 : 5	—	—
22 : 6	—	—
Cholesterol (g/100g of stone)	21.0	88.6

esterified fatty acids were higher in animals fed fat-free sucrose diets than in those fed various other diets, but the arachidonic acid level and the ratio of essential fatty acid to saturated fatty acids and oleic acid were reduced. These changes were greater in hamsters than in rats.

Cholesterol stones were found frequently in hamsters fed sucrose fat-free diets. Hamsters fed starch as the source of carbohydrate did not form any type of gallstones.

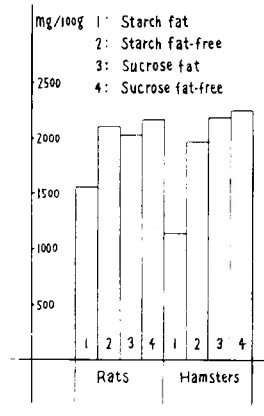
The concentration of total bile acids in the bile of hamsters fed fat-free glucose diets calculated by our colleague TANIMURA was 56.2 mg/dl, while in hamsters fed fat-free starch diets it was 79.3 mg/dl. In hamsters fed fat-rich starch diets it was 77.15 mg/dl, and in those fed fat-rich glucose diets and injected with pyridoxal phosphate it was 79.0 mg/dl. (Fig. 1, 2, 3, 4, 5.)



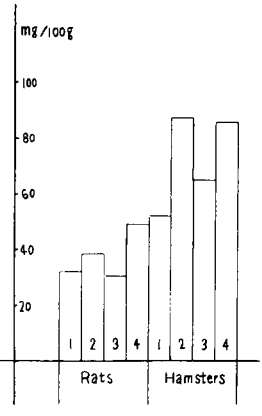
Total Cholesterol Concentration in the Liver

Esterified Cholesterol Concentration in the Liver

Fig. 1.

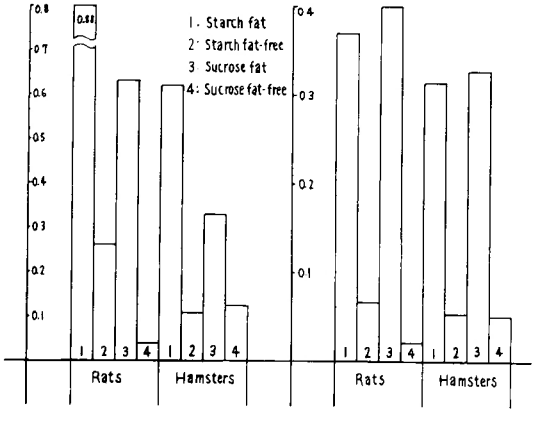


Total Fatty Acid Concentration in the Liver



Esterified Fatty Acid Concentration in the Liver

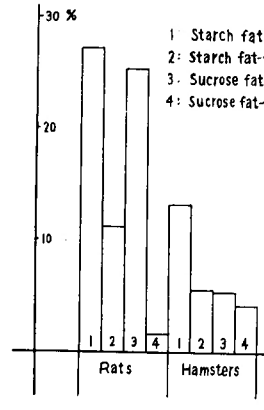
Fig. 2.



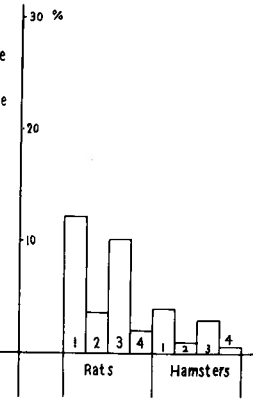
18 : 2+20 : 4/Saturated F. A. +18 : 1 in Total Fatty Acid

18 : 2+20 : 4/Saturated F. A. +18 : 1 in Esterified Fatty Acid

Fig. 3.



Percent of Total Arachidonic Acid



Percent of Esterified Arachidonic Acid

Fig. 4.

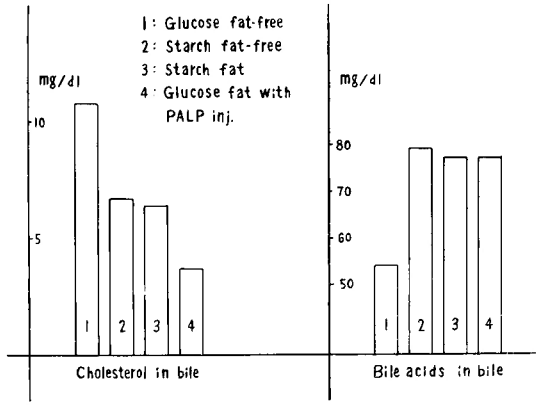


Fig. 5. Total Bile Acids and Cholesterol in Bile of Hamsters

V. DISCUSSION

Cholesterol, the main component of cholesterol stones, is insoluble in water by nature, so that other components are necessary to keep it in sloution in bile. Bile acids and phospholipids have been regarded as the substances taking part in keeping cholesterol in solution in bile. Many investigations on this problem have been carried out by WIELAND and others. They concluded that a colloid-chemical imbalance between bile acids and cholesterol was responsible for cholesterol precipitation. ISAKKSON considered that the ratio of bile acids to phospholipids, especially lecithin, was the most important factor in keeping cholesterol in solution in bile. MIYAKE maintained that the ratio of lecithin to cholesterol and of bile acids to cholesterol in bile should be higher than 6.6 and 8.0, respectively, in order to prevens cholesterol precipitation.

Human bile has a higher concentration of cholesterol and a lower concentration of bile acids and phospholipids than that of most animals, i. e. dogs and cows. Therefore, human bile is thought to favor the precipitation of cholesterol, since it is, so to speak, almost always completely saturated with cholesterol. Even a slight increase of biliary cholesterol may result in precipitation in man. The bile constituents of hamsters resemble those of man. Far from producing cholesterol stones, dogs actually dissolve stones in their gallbladders, as is known from the solubility test of human gallstones. Therefore, even a slight and temporary increase in the cholesterol level in bile must be an important factor in gallstone formation. Abnormalities in bile constituents can presumably be induced by disturbances of liver metabolism, to say nothing of biliary inflammation or billary stasis.

MATSUO, DREW, TEPPERMAN, DAM et al. have succeeded in the alimentary production of gallstones in hamsters or rabbits and have investigated extensively the details of bile composition. MARUYAMA and BLOMSTRAND found that the ratio of bile acids to cholesterol in the bile of patients with cholesterol stones was much lower than in those with other diseases.

It is well known and generally accepted that cholesterol is catabolized mainly to bile acids and sex- and adrenocortical-hormones. Recent investigations made by WIELAND,

SIPERSTEIN and BLOCH have clarified the biosynthetic pathway of cholesterol. (acetyl-CoA → hydroxy-methyl-glutaryl-CoA → cholesterol)

In our laboratory, HIKASA et al. have long studied the specific activity of essential fatty acids (EFA) in cholesterol metabolism and have come to the conclusion that in the adrenals, the biosynthesis of adrenocortical hormones, one of the end-metabolites of cholesterol, is influenced more by the EFA content than by the choleeterol concentration itself. This concept arose from the fact that in a state of EFA deficiency and/or metabolic disturbances of EFA, the adrenal EFA content fell markedly so that the adrenocortical capacity was greatly reduced, though a large amount of EFA was contained in the adrenals, heart and liver under normal conditions. It is logical to believe that the degradation of cholesterol to bile acids in the liver may also be affected by EFA, in the same way as in the adrenals. Our colleagues HIRANO and FUKUDA found that the concentration of EFA, especially tetraenoic acid, was decreased in the livers of patients with gallstones. They also found that their adrenocortical capacity was lower than that of patients with other diseases or of normal subjects.

SHIODA produced experimental cholesterol stones in hamsters by feeding mainly fat-deficient diets in which glucose or sucrose was the source of carbohydrate.

As described in the results, the quantity of total cholesterol in the livers of rats and hamsters was higher when fat-free sucrose diets were used than when fat-rich starch diets were given. Quantitative and qualitative analysis of liver fatty acids revealed the highest concentration in animals receiving a sucrose fat-free diet; however, it seems most likely that the net increase was brought about by the increase of saturated fatty acids plus oleic acid was lowest in animals on a sucrose fat-free diet. The difference in the ratio of EFA to saturated fatty acids and oleic acid between animals on a starch fat-rich and those on a sucrose fat-rich diet was greater than between those on a starch fat-free and a sucrose fat-free diet, since in those on fat-free diets, EFA in the liver decreased extremely, so difference in the ratio of EFA to saturated fatty acids and oleic acid could hardly be demonstrated between those on sucrose and on starch diets.

J. F. MEAD found that arachidonic acid is synthesized only from linoleic acid in the body. The influence of pyridoxine (Vitamin B_6) on this process has been studied by many investigators. HOLMAN first reported that rats deficient in both pyridoxine and EFA synthesized less arachidonic acid than those with simple EFA deficiency and that the addition of pyridoxine to their diet improved the conversion. Recentry many reports on the influence of pyridoxine have appeared, and controversy has continued as to whether such a correlation exists. In the present study, the ratio of arachidonic acid to linoleic acid in rats fed a pyridoxine-free diet differed from that in those on a control diet. But in hamsters no significant change was evident, and only slight changes in liver total cholesterol levels were observd. Though the composition of fatty acids did not change, the amounts of esterified fatty acids and cholesterol decreased in both rats and hamsters fed a pyridoxine-free diet, suggesting a disturbance in esterification. However, it is not clear whether the ingested pyridoxine was completely converted into its active form or not.

Thus, in rats and hamsters on fat-free diets, especially when sucrose or glucose was the source of carbohydrate, the concentration of cholesterol in bile and liver rose, the ratio

of EFA to other fatty acids was greatly decreased, bile acid and lecithin in bile diminished and a high incidence of cholesterol stone formation occurred. These findings led to the following conclusions.

1) The glycolysis of rapidly absorbed glucose or sucrose is performed probably by passing through a hexose-monophosphate shunt (HMP shunt) which may be activated in consequence of an adaptation or an urgent metabolic reaction in the liver, affecting some certain enzyme systems. Furthermore, glycolysis via the HMP shunt may also be promoted or activated under fat-free or fat-deficient conditions. Glycolysis via the HMP shunt presumably leads to an increase in biosynthesis of cholesterol and fatty acids other than EFA and, further, to a decrease in the biosynthesis of bile acids. As a matter of fact, under these dietary conditions, the concentration of liver fatty acids was higher and body weight gain was greater than in adult hamsters on a fat-rich starch diet. On the basis of the results of the studies reported by A. M. COHEN, C. COHN, PIGMAN, DAM et al., the following conclusions were reached. When animals received glucose or sucrose as the source of carbohydrate instead of starch or when animals were forced to ingest a high carbohydrate diet by stomach tube, glucose-6-phosphate dehydrogenase activity was elevated and the concentration of carbon dioxide in air expired soon after ingestion was increased, These results showed, presumably, that glycolysis under these conditions occurred via the HMP oxidative shunt. According to the studies of SIPERSTEIN et al., glycolysis through the HMP shunt activates the TPNH generating system so that the biosynthesis of cholesterol and fatty acids in the liver is accelerated. PAVEL maintained that the relatively reduced activity of DPNH due, perhaps, to glycolysis occurring through the shunt was possibly responsible for the decreased biosynthesis of bile acids. WIELAND repored that stimulation of TPNH activity enhanced cholesterolgenesis which competed with ketogenesis. This concept seems to be confirmed by the reports of L. VILLA et al. of increased concentration of cholesterol and decreased concentration of acetoacetic acid in the livers of patients with cholesterol stones.

2) In the livers of healthy hamsters, the concentration of arachidonic acid, particulary cholesterylarachidonate, was much lower than in those of rats. Further marked decreases of cholesterylarachidonate and cholesteryllinoleate were observed in hamsters fed a fat-free sucrose diet. Under these conditions, the catabolism of cholesterol to bile acids in the liver might be disturbed in the same way as in the adrenals. This metabolic disturbance was presumably accompanied by accumulation of cholesterol in the liver, leading to a further increase of cholesterol concentration and a decrease of bile acids in the bile. Already we have maintained that EFA, especially arachidonic acid, influenced the activation of the metabolic pathway of cholesterol to bile acids in the liver. We have also felt that the ratio of EFA, especially arachidonic acid, to other fatty acids in the liver has an important significance in cholesterol catabolism; that is the co-existence of saturated fatty acids in the liver disturbed the metabolic activity of FFA by means of, for instance, their high rate of consumption of vitamin B_6. So a true factor for gallstone formation may exist in the lowering of the ratio of EFA to saturated fatty acid.

To investigate this theory, the per cent of arachidonic acid in the total fatty acids was estimated. The liver of healthy men contained almost 11% arachidonic acid, and of

normal hamsters almost 13%. In patients with gallstones the arachidonic acid content of the liver was 8 %. In hamsters, it was 6% one week after the start of feeding a fat-free sucrose diet, and gallstones were already present in their gallbladders. Two weeks after ths start of feeding a fat-free sucrose diet, the liver arachidonic acid content was 3%. After 5-6 weeks, the liver arachidonic acid level rose somewhat. In these hamsters, cholesterol stones were frequently found. Therefore, it appears that when the arachidonic acid content of the liver is below 8% gallstone formation is apt to occur. (Table 12)

Table 12 Percentage of Each Fatty Acid in the Liver of Patients with Cholesterol Stones and Hamsters on Fat-free Sucrose Diet.

	Liver of Men		Liver of Hamsters			
	Control	Patients with cholesterol Stone	Control Diet	After the Start of Feeding a Fat-free Sucrose Diet		
				1 week	2 weeks	5～6 weeks
16 : 0	24	23	20.9	18.9	18.4	19.7
16 : 1	2.5	4.5	1.1	9.5	5.3	4.2
18 : 0	17	13	19.4	6.4	9.6	10.6
18 : 1	12.5	19	17.4	40.6	45.8	38.2
18 : 2	16	16.5	22.6	7.8	3.9	9.3
18 : 3	0.5	0.6	0	0	0	0
20 : 3	1.0	1.1	0.1	2.5	8.9	4.9
20 : 4	11.0	7.8	13.4	6.0	3.4	5.6
22 : 0	2.1	1.3	0.3	—	—	—
22 : 6	11	9	2.4	5.5	3.9	5.6

3) H. DANIELSSON reported that in animals which were reared under germ-free conditions, the cholesterol level in the serum and in the liver was higher and the bile acids in the intestines were more slowly catabolized than in control animals, because there was no destruction of bile acids by the intestinal flora. Therefore, more bile acids returned to the liver from the intestines. Consequently less biosynthesis of bile acids took place in the liver, leading to an accumulation of cholesterol in the serum. The animals receiving fat-free glucose or sucrose diets may more closely resemble animals reared under germ-free conditions than those on a fat-rich starch diet. Multiplication of flora in the lower intestine may be disturbed for want of nutrients, because glucose and sucrose are now thought to be quickly absorbed from the upper part of the intestine. A decrease in the daily production of bile acids must be accompanied by accumulation of cholesterol in the liver, then in the bile. These changes are probably quickly controlled and repaired by a feed-back mechanism, but the bile-equilibrium may occasionally be upset. Once precipitated cholesterol may grow into gallstones by secondary factors.

Pyridoxine, inositol, biotin and vitamin K, which are synthesized by the intestinal flora, may be considerably affected by various dietary conditions. The reeuction in vitamins may disturb cholesterol catabolism to bile acids. In hamsters fed a vitamin B_6 deficient sucrose diet containing fat, pigmented stones only were produced, and cholesterol stone formation was complstely prevented.

Recently, the in vivo metabolic action of inorganic magnesium has been studied.

BEHRSOHN observed a negative correlation between serum cholesterol and serum magnesium levels. VITAL et al. observed that magnesium deficiency in dogs caused lipid deposition in the heart. However, in the present study, there was little or no difference in cholesterol levels or composition of fatty acids in the liver between magnesium-deficient rats and controls. Diarrhea, which occurred as a complication of magnesium deficiency, might hinder the absorption of ingested lipids, even though a little decrease of the linoleic acid level in the liver was observed.

HASHIMOTO, one of our colleagues found no correlation between the calcium level and the magnesium level in the bile of rats. Moreover, he found no gallstones of any type in hamsters fed fat- and magnesium-free starch diets. Thus, inorganic magnesium seems to play no important role in gallstone formation.

In 1930 SWEET suggested that the "Liesegang Phenomenon" might be a factor in gallstone formation. Therefore, we wondered whether large amounts of silicic acid in the diet might raise the concentration of silicic acid and inorganic calcium in the bile and whether the level of cholesterol in the liver might also be increased simultaneously. However, TANIMURA and HASHIMOTO found that the levels of silica and calcium were not elevated in the bile of rats receiving large doses of silicic acid. In this study, the levels of cholesterol and fatty acids in the livers of these rats showed little change.

VI. SUMMARY AND CONCLUSION

1) Cholesterol and fatty acids in the livers of rats and hamsters fed various diets were analysed and their relationship to gallstone formation, especially cholesterol stones, was studied.

2) Cholesterol stones were formed in the gallbladders of hamsters fed fat-free diets in which sucrose or glucose was the source of carbohydrate. In these animals, the levels of cholesterol and total fatty acids, especially saturated fatty acids, in the liver were the highest. The levels of essential fatty acids, especially cholesterylarachidonate, in the liver was greatly reduced in these hamsters.

3) The ratio of EFA to other fatty acids in the liver was much lower in animals on sucore diet than in those on a starch diet.

4) Animals deficient in pyridoxine showed lower levels of fatty acid combined with cholesterol in the liver.

5) The acceleration of choleserol biosynthesis in the liver was thought to be a necessary factor for cholesterol stone formation, together with disturbances of cholesterol catabolism to bile acids.

6) No changes were detected in the levels of cholesterol and fatty acids in the livers of magnesium-deficient rats. There appears to be no correlation between magnesium deficiency and cholesetrol metabolism.

7) Feeding of a diet high in silica had no influence on cholesterol or fatty acid metabolism.

8) The levels of EFA in human gallstones were very low both in cholesterol stones and bilirubin calcium stones. Bilirubin calcium stones contained approximately five times the amount of total fatty acids as cholesterol stones.

I wish to express my sincerest gratitude to Assistant Professor Dr. YORINORI HIKASA for his continual guidances and encouragements during the course of this study.

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和文抄録

コレステロール系胆石の発生原因，特にコレステロール代謝の態度に関する研究

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江口　隆

われわれの教室では従来より，不可欠脂肪酸(EFA)と副腎皮質機能との間に密接な関係があることを認めて来た．すなわち，コレステロールから副腎皮質ホルモンへの代謝過程において，コレステロールはEFA，特にテトラエンとエステル結合していることが必要であり，このエステルが減少しているときは副腎皮質機能が低下していることを認めた．福田，平野等はまた，胆石患者の副腎皮質機能が低下し，同時に血清および肝臓中のアラキドン酸量の低下があることを認めた．又塩田等はハムスターにグルコースを糖質源としたEFA欠乏食を投与することにより実験的に胆石を作製し，その胆汁中の胆汁酸，レシチンが減少し，コレステロールが増量していることを認めた．以上の事実より，コレステロール結石症の場合，コレステロールからその一方の代謝産物である胆汁酸への移行が副腎におけると同様にEFA欠乏により障害をうけているものと推測され得る．

胆石，殊にコレステロール系結石の発生に関して肝臓中のEFAおよびコレステロールの態度をラッテ，ハムスターおよび人胆石を用いてその遊離型およびエステル型について検討し，さらにコレステロールの生体内合成に関しても考察を加えた．

1）ラッテおよびハムスターに砂糖を糖質源とした合成食を投与すると，糖質源を澱粉とした場合よりも総脂肪酸，総コレステロール共に増量した．そのエステル型には大きな変化がなかつた．又総脂肪酸中，EFAの占める割合も後者では著しく低下しておりエステル型における割合は変化しなかつた．

以上の点から，砂糖食においては澱粉食と比較して相対的EFA欠乏状態にあると考えられる．

2）一方無脂肪食で，糖質源を砂糖にすると，ハムスターではその胆嚢中にコレステロール結石の発生をみるが，その肝臓中コレステロールおよび脂肪酸は総量およびエステル量共に著明に上昇するが，脂肪酸中EFAの占める割合は著明に低下する．特にハムスターにおけるアラキドン酸量は微量であつた．

全脂肪酸に対するアラキドン酸の占める割合は，胆石発生ハムスターおよび人間胆石症患者とも8％以下であつた．

3）ビタミンB_6欠乏食ラッテおよびハムスターでは特にEFAの減少は認められず，またリノール酸に対するアラキドン酸の割合も対象と比較して特に低い値は認められなかつた．ただエステル型脂肪が全体的に減少しており，エステル化の障害を推測させた．

4）マグネシウム欠乏食ラッテおよびシリカ過剰食ラッテにおいては肝臓中コレステロールおよび脂肪酸量に特に変化は認められず，また胆石発生との関係も見出し得なかつた．

5）人間の胆石中に含有される脂肪酸を分析し，コレステロール系石，ビリルビンカルシウム系石ともにEFAの含量が著しく少いことを認めた．

6）砂糖無脂肪食ハムスターでコレステロール系結石の発生をみるが，これは砂糖が澱粉に比較して吸収が早いためそのグリコリーシスがHexose-Monophosphate Shuntを通り，その結果脂肪酸およびコレステロールの生合成が高まり，過剰のコレステロールが胆汁中に排泄されると考えられる．さらに無脂肪の状態がこのShuntの活性を高めると推測される．

いいかえれば，コレステロール系結石発生に関しては，コレステロールの生合成亢進と，その胆汁酸への代謝障害がともに必要な条件であると考える．